Characterization of Microbial Communities Found in Bioreactor Effluent

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Abstract

The purpose of this investigation was to examine microbial communities of simulated wastewater effluent from hollow fiber membrane bioreactors collected from the Space Life Science Laboratory and Texas Technical University. Microbes were characterized using quantitative polymerase chain reaction where a total count of bacteria and fungi were determined. The primers that were used to determine the total count of bacteria and fungi were targeted for 16S rDNA genes and the internal transcribed spacer, respectively. PCR products were detected with SYBR Green I fluorescent dye and a melting curve analysis was performed to identify unique melt profiles resulting from DNA sequence variations from each species of the community. Results from both the total bacteria and total fungi count assays showed that distinct populations were present in isolates from these bioreactors. This was exhibited by variation in the number of peaks observed on the melting curve analysis graph. Further analysis of these results using species-specific primers will shed light on exactly which microbes are present in these effluents. Information gained from this study will enable the design of a system that can efficiently monitor microbes that play a role in the biogeochemical cycling of nitrogen in wastewater on the International Space Station to assist in the design of a sustainable system capable of converting this nutrient.

Nomenclature

HF-MBR	=	hollow fiber membrane bioreactor
ITS	=	internal transcribed spacer region
PCR	=	polymerase chain reaction
qPCR	=	quantitative polymerase chain reaction
rDNA	=	ribosomal deoxyribonucleic acid
SLSL	=	Space Life Science Laboratory
TBE	=	tris/borate/ethylenediaminetetraacetic acid
Tm	=	melting temperature
TTU	=	Texas Tech University
dF/dT	=	derivative of the fluorescence as a function of temperature

I. Introduction

Nitrogen is a vital element found in organic compounds, such as proteins, nucleic acids, and chlorophyll. It is the most abundant gas in the Earth's atmosphere; however, it is has to be transformed into its usable form before it can be utilized. Microbes are the driving force behind the biogeochemical cycling of nitrogen. A variety of microorganisms play a role in the biogeochemical dynamics of the nitrogen cycle, such as bacteria, archaea, and fungi. The major conversions of nitrogen are nitrogen fixation, nitrification, denitrification, anaerobic ammonia oxidation (anammox), and ammonification.[1]

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Hollow fiber membrane bioreactors (HF-MBR) are mechanical devices that are used to cultivate microbes that play a role in the biological conversion of materials. These devices are used at wastewater treatment plants to remove organic and inorganic contaminants.[2] Proper functioning of HF-MBR requires monitoring environmental conditions as well as characterization of the diversity of microbes involved in these processes to ensure that all of the transformations are in balance. A variety of techniques have been used to characterize microbial communities, such as selective plating, community-level profiling and fatty acid methyl ester analysis to name a few. [3]

The aim of this study was to use quantitative PCR to determine that total count of bacteria and fungi present in HF-MBR effluent and to visualize various populations of microbes via unique melting curve profiles. The assay was based upon the ability of this approach to detect sequence variation present within the 16S rDNA and the ITS amplicons using universal primers for each gene.

II. Materials and Methods

A. Effluent sampling

Fifteen milliliter triplicate aliquots of effluent from a HF-MBR at the Space Life Science Laboratory (R3 1, R3 2, and R3 3)) and HR-MBR at Texas Tech University (TTU 1, TTU 2, and TTU 3)) were collected on three different dates and stored at -80°C. The reactor at the Space Life Science Laboratory was fed ammonium bicarbonate, the TTU reactor was fed a proprietary blend.

B. DNA isolation

DNA was extracted from triplicate effluent samples in using UltraClean Soil DNA Isolation Kit (Mo Bio) according to the manufacturer's instructions. The isolated DNA was eluted in 50µl elution buffer and the DNA concentration was determined by using the Qubit 2.0 Fluorometer (Invitrogen) then stored at −20°C for further analysis.

C. Quantitative real time PCR

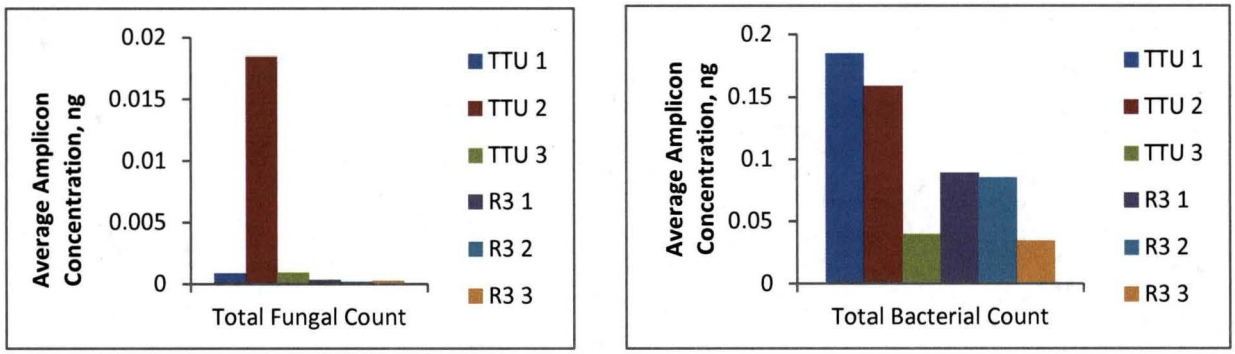
Total bacterial and fungal counts were determined by using 16S rDNA primers EUB338F (5'-ACTCCTACGGGAGGCAGCAG) and EUB518R (5'-ATTACCGCGGCTGCTGG) and ITS primers ITS1f (5"-TCCGTAGGTGAACCTGCGG) and 5.8S (5"-CGCTGCGTTCTTCATCG), respectively. Each bacterial reaction mixture (20µl total volume) consisted of 10µl SYBR Green I Master Mix (Roche), 3.6µl of water, 0.3µl of each primer (Invitrogen), 0.8µl bovine serum albumin (New England Biolabs), and 5µl of DNA. Fungal reactions (20µl total volume) consisted of 10 µl SYBR Green I Master Mix (Roche), 3.2µl of water, 0.5µl of each primer (Invitrogen), 0.8 µl bovine serum albumin (New England Biolabs), and 5µl DNA. qPCR conditions were 10 min at 95°C, followed by 35 cycles of 95°C for 1 min, 53°C for 30 sec, and 72°C for 1 min. Melting curve analysis conditions were 95°C for 30 sec and 65C for 30 sec. Five-fold serial dilutions of *Escherichia coli* DNA for bacterial counts and *Penicillium* for fungal were used to construct a standard curve. qPCR was performed in duplicate using an LC480 Lightcylcer (Applied Biosystems). PCR cycles were recorded through the SYBR Green (483–533 nm) channel using the default LC480 data acquisition settings. Melt curve profiles were assessed and analyzed using LightCycler 1.5.0.39 software.

To measure the concentration of amplicons produced, we used an external calibration curve generated by 5-fold serial dilutions of DNA containing the target gene. To check the assay specificity, we performed melting point analyses, and subsequently the PCR products were run in agarose gels. The melting point analysis was initiated at 95°C for 30 sec and a subsequent cooling step at 65°C for 30sec, followed by continuous sampling of the fluorescence signal until the temperature reached 95°C, with a temperature transition profile of 0.11°C/s. When the melting temperature (T_m) is reached, double stranded DNA is denatured and the SYBR Green1 is released which cause a dramatic decrease in fluorescence intensity. The rate of this change was determined by plotting the derivative of the fluorescence relative to the temperature (dF/dT) vs. temperature by data analysis software of the real-time PCR instrument. The temperature at which a peak occurs on the plot corresponds to the Tm of the DNA duplex. After the qPCR was completed, the amplicons of each sample were run in 2% agarose gels containing SYBR Safe (Invitrogen) diluted 1:10,000 in 0.5 X TBE and visualized using a FluorChem 9000™ imaginer (Alpha Innotech).

III. Results

Quantitative PCR results showed that the total bacteria count of effluents from both bioreactors exceeded the total fungal counts as illustrated in Fig. 1. The overall concentration of amplicons produced for total bacteria and total fungi was 0.09841ng and 0.00349ng, respectively.

16S rDNA amplicons generated from duplicate reactions of three isolates from each bioreactor were subjected to a melting curve analysis. Results indicate the presence of at least two distinct amplicon populations in effluent samples collected from TTU. Samples collected from the SLSL bioreactors exhibited only one peak Fig. 2. To confirm the size of the amplicon, the PCR products were run on a 2% agarose gel (data not shown). The average T_m of the two peaks produced by TTU showed the presence of populations that differed by ~7.7°C. The average T_m of R3 effluent samples produced a single melting peak at approximately 82.2°C. T_m averages for 16S rDNA amplicons are summarized in Table 1.



a) b)

Figure 1. Quantitative PCR. a) 16S ITS1f/5.8S amplicons and b) 16S rDNA amplicons.

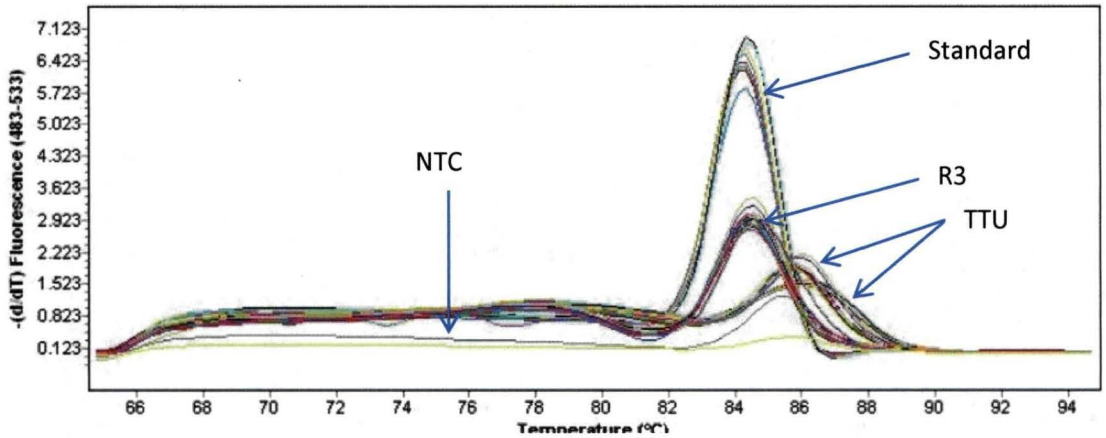


Figure 2. Melting Curve Analysis of Bacterial Community.

Table 1. Average Tm of 16S rDNA amplicons.

	Average T_m °C		SD	
Sample Name	**Peak 1**	**Peak 2**	**Peak 1**	**Peak 2**
TTU 1	77.8	86.3	0.50	0.08
TTU 2	78.1	86.1	0.42	0.26
TTU 3	79.1	85.8	0.51	0.09
R3 1	84.7	-	-	-
R3 2	81.9	-	-	-
R3 3	80.2	-	-	-

Melting curve analysis of ITS1f/5.8S amplicons from fungal DNA extracts also indicated the presence of different populations Fig. 3. It was observed that the TTU bioreactor produced amplicons with an average T_m of ~80°C. There was also a second peak observed for the TTU bioreactor which had an average Tm of 86.8°C. Two

melting peaks were observed in all three R3 isolates at ~ 82°C and ~87°C. T_m averages for 16S rDNA amplicons are summarized in Table 2.

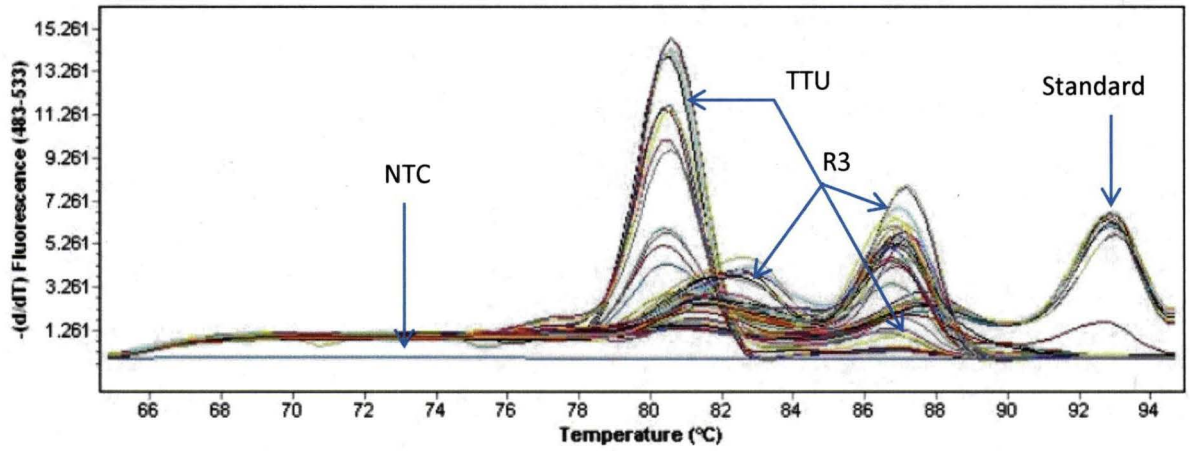


Figure 3. Melting Curve Analysis of Fungal Community.

Table 2. Average Tm of ITS1f/5.8S amplicons.

	Average T_m °C		SD	
Sample Name	**Peak 1**	**Peak 2**	**Peak 1**	**Peak 2**
TTU 1	80.7	86.8	0.32	0.15
TTU 2	80.6	-	0.06	-
TTU 3	80.5	-	0.07	-
R3 1	82.4	87.0	0.28	0.12
R3 2	82.1	87.0	0.58	0.21
R3 3	81.9	87.02	0.47	0.13

IV. Conclusion

Melting curve analysis is a useful technique in the identification of organisms in a community. This approach analyzes the shape of the melting curve of an amplicon, which provides more information than the melting temperature alone. Short, regional sequences denature to form single stranded regions, which release double-stranded DNA-binding fluorescent dyes, before reaching the temperature at which the entire amplicon denatures. This influences the shape of the melt curve, to generate unique curves that reflect species-specific sequence differences. This has enabled identification of bacterial and viral species when compared to known T_m.[4] Not only does this technique allow rapid analysis of microorganisms in a community, but it also does not require the design of species-specific primers.

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